Role of lymphotoxins in chronic inflammatory kidney diseases, kidney transplant rejection and urinary prion excretion

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HABILITATIONSSCHRIFT

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Content

Abbreviations

AR	acute rejection
BAFF	B cell activating factor
BR	borderline rejection
cABMR	chronic antibody mediated rejection
CNS	central nervous system
DC	dendritic cell
DcR3	decoy receptor 3
dpi	days post inoculation
FDC	follicular dendritic cell
GN	glomerulonephritis
HVEM	herpes-virus entry mediator
i.p.	intraperitoneally
IgAN	immunoglobulin A nephritis
LD50	median lethal dose
LIGHT	LT-like exhibits inducible expression and competes with herpes simplex virus glycoprotein D for herpes-virus entry mediator
LN	lupus nephritis
LT	lymphotoxin
LTβR	lymphotoxin beta receptor
MBA	mouse bioassay
MFGE8	milk fat globule-EGF factor 8 protein
MHC	major histocompatibility complex
NF-κB	nuclear factor kappa-light-chain enhancer of activated B cells
NZB	New Zealand black mouse
NZBW	New Zealand black x New Zealand white F1 generation
NZW	New Zealand white mouse
PrP	prion protein
PrP^{C}	prion protein cellular = cellular prion protein
PrP^{Sc}	prion protein scrapie = pathogenic (misfolded) form of the prion protein
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RIP	rat insulin promoter
SLC	secondary lymphoid-organ chemokine
SLO	secondary lymphoid organs
sLTβR	soluble lymphotoxin beta receptor
TEC	tubular epithelial cell
TLO	tertiary lymphoid organs
TLT	tertiary lymphoid tissue
TNF(R)	tumor necrosis factor (receptor)

1. Introduction

Lymphotoxins (LTs) are cytokines, which belong to the TNF superfamily. Two distinct forms have been identified designated lymphotoxin α (LTα)[1, 2] and lymphotoxin β (LTβ)[3]. They are part of a multifaceted signaling network[4, 5]. LTα exists as secreted homotrimer (LTα3) or in conjunction with LTβ as a membrane-anchored heterotrimer LTα1β2 or LTα2β1, the latter of which is less abundant[3, 6]. Ligands overlap in binding to three related receptors termed TNF receptor 1 (TNFR1), TNF receptor 2 (TNFR2) and the lymphotoxin β receptor (LTβR). LTα3 signals via TNFRs 1 (and 2)[7], whereas LTα1β2 only binds to LTβR[8].

LIGHT (LT-like exhibits inducible expression and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator) is another member of the TNF family[9, 10] and homologous to LTs[11]. It is membrane anchored and can bind to the LTβR[12]. Another receptor for LIGHT is HVEM (herpes-virus entry mediator), which is expressed on T cells[13]. Furthermore, LIGHT can also bind to Decoy receptor 3 (DcR3) (**Figure 1**), which is a secreted receptor that blocks the biologic action of the ligands FasL and LIGHT[14, 15] but can also activate T cells via binding to TL1A[16].

Activation of LTβR via LIGHT or LTα1β2 can activate the classical and alternative nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) pathway promoting inflammation, immune cell clustering and secondary lymphoid organ (SLO) formation[4, 17] (**Figure 1**). LTβR is ubiquitously expressed in parenchymal cells (stromal fibroblasts, epithelial cells), follicular dendritic cells, endothelial cells (ECs) and cells of myeloid origin, but not by T, B or NK cells[18-21]. It is strongly expressed in the mouse kidney[22]. The LTα1β2-LTβR system controls the organogenesis and homeostasis of SLO[4]. The LTβR is involved in T cell costimulation and clonal expansion of T cells and important for maintenance and proliferation of dendritic cells (DCs)[23-26].

LT signaling plays a significant role in chronic inflammation[27] and is important in the formation of tertiary lymphoid organs (TLOs)[28-31] in a process termed lymphoid neogenesis[27]. In renal biopsies with chronic glomerulonephritis (GN) or interstitial nephritis, TLOs are detected in over 25% of biopsies and in renal allografts in up to 50%[32-34]. LTα and LTβ are expressed on blood B, T and NK cells upon activation[35, 36]. Within lymphoid organs, LTs are constitutively expressed on lymphocytic cells and DCs, consistent with a maintenance function in these tissues[37-39]. Nonhematopoietic cells such as hepatocytes[40, 41], glial cells[42] and multinucleated giant cells also express LTα and β[43].

Experimental therapeutic strategies have been developed to interfere with LT signaling. Browning and colleagues have created a soluble LTβR-Ig (sLTβR-Ig) fusion protein, which blocks signaling of LTα1β2 and LIGHT[44]. Furthermore, an LTα antibody has been created, which antagonizes LTα3 and LTα1β2[45]. Blocking LT signaling using these approaches has been therapeutic in non-renal autoimmune inflammatory

conditions in animal models such as collagen-induced arthritis and experimental autoimmune encephalitis[45], Sjoegren's syndrome[46], colitis[47] and insulitis[48]. In experimental prion disease, treatment with sLTβR-Ig abolishes prion accumulation and replication in the spleen and retards neuroinvasion of the infectious agent[49, 50].

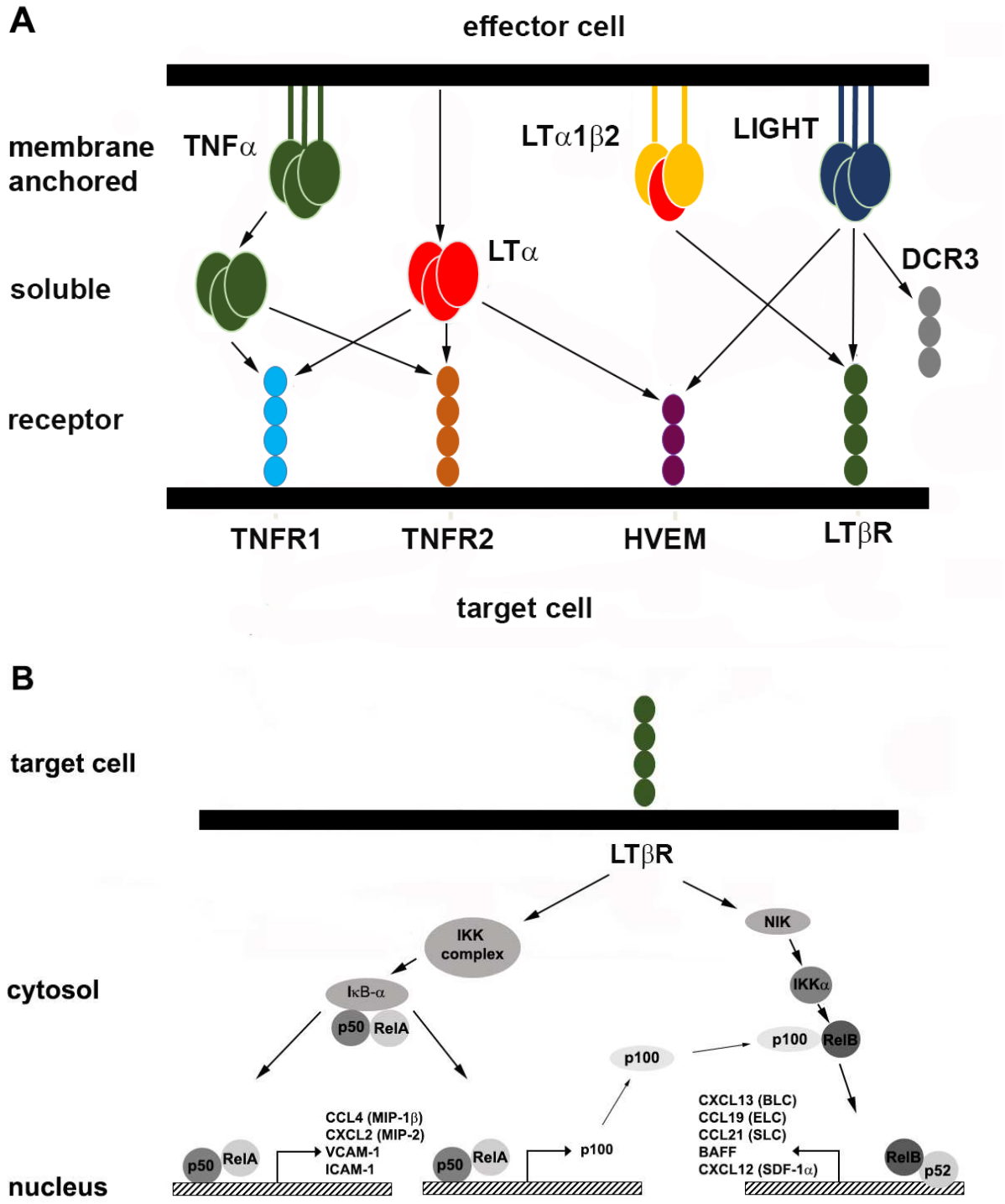

Figure 1. (**A)** Schematic representation of the receptors and ligands of the TNF/LT family. (**B)** Upon ligation of the LTβR, two NF-κB pathways can become activated. The canonical (left) results in initiation of IKKβ and RelA and stimulates expression of inflammatory genes like VCAM-1, CCL4, and CXCL2. Additionally, it triggers an increase of the NF-κB2/p100 precursor

protein. The alternative or non-canonical pathway (right) involves the stimulation of NIK, which activates IKKα to generate active p52, which together with RelB triggers the transcription of genes associated with organogenesis and homeostasis of SLOs and TLOs such as CXCL13, CCL19, BLC, CCL21, CXCL21 and BAFF (adapted from[5, 17, 51]).

Even though LTs play an important role in chronic inflammation with or without formation of TLOs, barely any information is available concerning the LT system in kidney diseases. We therefore investigated renal LT expression in inflammatory kidney diseases such as IgA nephropathy, lupus nephritis and crescentic glomerulonephritis in humans. Furthermore, we examined the expression of LTs in human kidney biopsies with acute and chronic allograft rejection and in a rodent model of acute transplant rejection. To assess the functional role of LTs in kidney inflammation we studied the effects of LTβR signaling in renal cells in vitro and the impact of blocking the LTβ receptor in a rodent model of lupus nephritis. Since LTs had been shown to be important in peripheral prion propagation in SLOs we explored the impact of LT induced and spontaneous inflammation in the kidney and other parenchymal organs on peripheral prion pathogenesis in mice.

2. Summary of included publications

2.1. Chronic lymphocytic inflammation specifies the organ tropism of prions[52]

Chronic inflammatory conditions typically lead to infiltration of the affected tissue with inflammatory cells. Infiltrating cells may be diffusely scattered but can also form larger aggregates with lymphoid microarchitecture. These organized structures are called TLOs or tertiary lymphoid tissue (TLT)[31]. As described above, the LT system is important in TLO formation. Organized lymphoid infiltrates comprise T and B lymphocytes, follicular dendritic cells (FDCs), DCs, and macrophages[32, 53-55]. FDCs, B cells and other components of the immune system are also involved in prion propagation[49, 56-60]. Prions are proteinaceous infectious agents causing slowly developing fatal central nervous system (CNS) diseases called transmissible spongiform encephalopathies (TSEs)[61] such as Creutzfeldt-Jakob disease in humans and scrapie in sheep. Prions are detectable in SLOs (i.e. spleens, lymph nodes, peyer's patches) long before they invade the CNS and cause clinical symptoms[62]. Non-neural and non-lymphoid tissues are usually devoid of prions. PrP^{Sc} is the misfolded disease associated isoform of the normal host prion protein (PrP^{C}) and accumulates in the CNS and SLOs of affected animals[61, 63, 64]. As opposed to PrP^{C}, PrP^{Sc} is insoluble in detergents and relatively resistant to digestion with proteases. Some researchers use the term PrP^{Sc} synonymous with prion infectivity[61]. Besides PrP^{C}, replication of prions in SLOs necessitates follicular dendritic cells (FDCs). Maintenance of FDCs depends on LT α, β and TNF[56-58, 65, 66]. Inhibition of LT and TNF interferes with peripheral prion replication and neuroinvasion[49, 67-69]. We thus hypothesized that chronic inflammation with upregulation of LTs in non-lymphoid organs might influence prion pathogenesis. We scrutinized this question in transgenic and spontaneous mouse models of chronic inflammation, including nephritis, pancreatitis, and hepatitis. As a model for renal inflammation, we selected transgenic mice expressing LTα under the control of the rat insulin promoter (RIP) in pancreatic β islet cells and renal proximal tubules[28, 70]. These animals develop interstitial inflammatory follicles with features of TLOs in kidney and pancreatic islets. As an additional model for chronic renal inflammation, we chose NZB x NZW-F1 (NZBW) mice. Besides glomerulonephritis NZBW mice develop interstitial nephritis with distinct B and T cell areas, small FDC-clusters, and features indicative of functional germinal centers. Parental NZW mice are devoid of interstitial nephritis and were used as controls. We furthermore investigated mice expressing the secondary lymphoid organ chemokine (SLC = CCL21) under control of the RIP[71]. These mice (RIPSLC) contain follicles in the pancreas with organized T and B cell zones and FDC networks. As a chronic hepatitis model, we used bitransgenic mice expressing LTα and β in the liver under the control of the albumin promoter. Livers of AlbLTαβ mice have structured aggregates of inflammatory cells with features of TLOs. Quantitative RT-PCR (qRT-PCR) revealed that LTβ was

significantly upregulated not only in LT transgenic, but also in NZBW kidneys whereas RIPSLC mice had increased LTα expression in pancreas and kidney.

AlbLTαβ, RIPLTα, NZBW, NZW, RIPSLC and control mice were inoculated intraperitoneally (i.p) with a dose of 5 log LD50 mouse scrapie prions (LD50 = median lethal dose) and sacrificed at various days post inoculation (dpi). PrP^{Sc} tissue loads were determined by Western blotting of organ homogenates after enrichment by phosphotungstate precipitation[72, 73]. We detected PrP^{Sc} at 60, 75, and 90 dpi in comparable amounts in all spleens of each genotype, but not in livers, kidneys, or pancreases of wild-type mice. At 75 dpi PrP^{Sc} immunoreactivity was detected in two out of three AlbLTαβ livers while RIPLTα kidneys and pancreases were negative. At 90 dpi, PrP^{Sc} was present in all RIPLTα kidneys, RIPLTα pancreases, AlbLTαβ livers, and NZBW kidneys. With the exception of one RIPLTα kidney, which disclosed possible traces of PrP^{Sc}, control kidneys, pancreases and livers were negative. By histoblotting of tissue, we confirmed PrP^{Sc} deposits colocalizing with inflammatory infiltrates in inflamed organs.

We then investigated whether inflammation influences the distribution of prion *infectivity* during the preclinical phase of infection. Tissue homogenates of prion inoculated mice were assayed at 60 dpi for prion infectivity by mouse bioassay (MBA; incubation time interval assay)[74, 75]. Spleens displayed comparably high titers of 4 - 6 log LD50 prion infectivity per gram of tissue. Wild-type pancreata and kidneys lacked infectivity, whereas RIPLTα kidney and pancreas titers ranged between borderline (i.e. < 100% of indicator animals inoculated developed prion disease) and 1.4 log LD50/g. At 75 dpi, RIPLTα pancreas and kidney titers were 3.3 and 4 log LD50/g, whereas controls were noninfectious. At 90 dpi, all RIPSLC and RIPLTα pancreata and one RIPLTα kidney had prion titers approximating those of spleen, while controls displayed undetectable or borderline infectivity. NZBW kidneys contained prion titers of 2.5 to 3.5 log LD50/g whereas controls were noninfectious. Infectivity of wild-type livers, kidneys, and AlbLTαβ kidneys was below detectability or borderline, while AlbLTαβ livers had titers of 3.1 to 3.4 logLD50/g 90 days dpi (**Figure 2**).

LTα-/- and LTβR-/- knockout mice suffer from spontaneous structured lymphocytic infiltrations in liver, kidney, pancreas, lungs and other organs[76-78]. The inflammatory foci contain B, T and dendritic cells, but lack mature FDCs. Despite the severe inflammatory changes livers and kidneys of prion-inoculated LTα-/- and LTβR-/- mice were devoid of prion infectivity or PrP^{SC} at 90 dpi.

Taken together, this study was the first to indicate, that chronic follicular inflammation permits accumulation of prion infectivity in otherwise prion-free organs such as kidney, pancreas and liver.

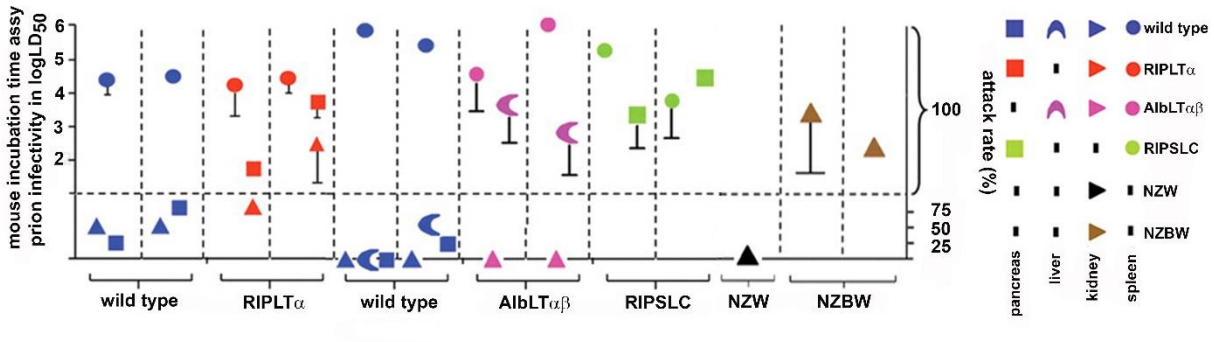

Figure 2. Prion infectivity in organs with and without inflammation. Infectivity titers in organs of wild-type, RIPLTα, AlbLTαβ, RIPSLC, NZW and NZBW mice as quantified by transmission to indicator mice at 90 dpi. Each column demarcated by dashed lines indicates one mouse. Symbols underneath the dashed horizontal line designate attack rates of <100%. Apart from a RIPLTα kidney that caused an attack rate of 75%, RIPLTα and NZBW kidneys, RIPSLC and RIPLTα pancreases, and AlbLTαβ livers comprised prion infectivity with significant titers at 90 dpi. Wild-type kidneys, livers and pancreases and NZW kidneys contained borderline or undetectable prion infectivity (adapted from [52]).

2.2 Coincident scrapie infection and nephritis lead to urinary prion excretion[79]

Above we established that interstitial inflammation with TLO like structures in the mouse kidney induced by ectopic expression of the cytokine LTα enabled prion accumulation. Mice with lupus-like disease (NZBW mice) and organized inflammatory foci in the kidney (associated with upregulation of LTβ) also accumulated prions. In this study, we examined if prion infectivity is shed into the urine from kidneys replicating prions.

First, we performed extensive histologic analysis to characterize the renal phenotype of several mouse models we intended to use to address this question. We revealed that the ectopic expression of LTα in the kidney did not only lead to organized tubulointerstitial inflammation, but also resulted in a glomerular pathology. RIPLTα mice suffer from an immune complex glomerulonephritis with mesangial proliferation and glomerular subendothelial immunoglobulin (Ig) and complement deposition. NZBW mice have severe crescentic GN associated with glomerular immune complex and complement deposition with prominent glomerular capillary sclerosis. MFGE8 (milk fat globule-EGF factor 8 protein) knockout (-/-) mice[80], which are inflicted with circulating antinuclear and anti-dsDNA antibodies, showed glomerular basement membrane thickening, mesangial and endocapillary hypercellularity, glomerular immune complex and complement depositions with mesangial and subendothelial deposits on electron microscopy (EM). NZW mice had a mild glomerular phenotype with few mesangial electron dense deposits on EM. Histology of C57BL/6 and 129Sv x C57BL/6 mice was unremarkable. Only RIPLTα and NZBW mice exhibited organized

tubulointerstitial infiltrates (**Figure 3**). Compared to controls, NZBW, NZB and MFGE -/- mice were strongly, whereas RIPLTα and NZW only mildly proteinuric.

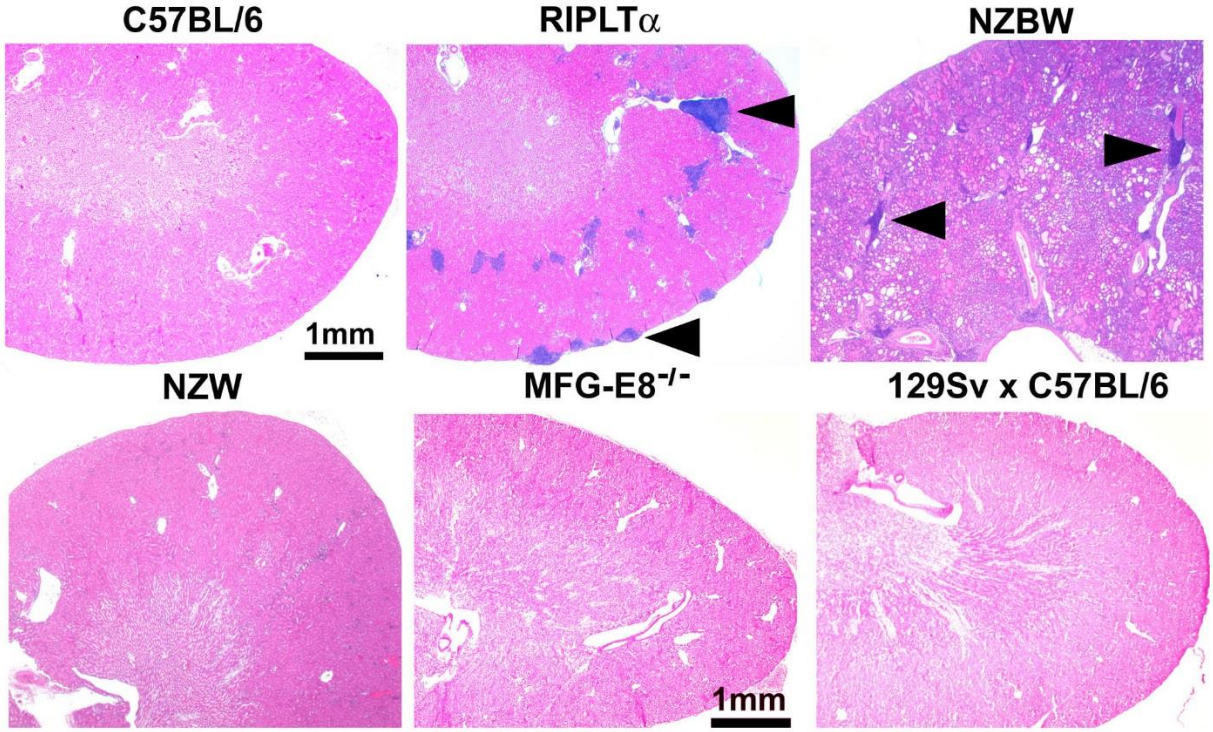

Figure 3. Histopathology in RIPLTα, NZBW, MFG-E8-/- and control mice. H&E-stained paraffin sections of age-matched RIPLTα, C57BL/6, NZBW, NZW MFG-E8-/- and 129Sv x C57BL/6 kidneys. Renal lymphocytic infiltrates are present in RIPLTα and NZBW (arrowheads), but not in MFG-E8-/- kidneys or controls (NZW, C57BL/6, 129Sv x C57BL/6).

To test if prion infectivity spills over from inflamed kidneys into urine, we administered prions i.p. to RIPLTα and NZBW mice. I.p. prion injected AlbLTαβ transgenic mice (no renal pathology), NZW, NZB, C57BL/6, tga20 transgenic mice (which overexpress PrP^C approximately 20 fold[74]), MFG-E8 knockout[80] mice and 129Sv × C57BL/6 served as controls since these mice do not develop interstitial nephritis. We collected urine from each genotype at various time points before the animals became symptomatic from prion disease and from terminally scrapie-sick mice. We intracerebrally inoculated purified urinary proteins pooled from groups of three to six mice into PrP^C overexpressing tga20 mice, which served as indicator animals (**Figure 4**). We detected prion infectivity in urine of presymptomatic RIPLTα and NZBW as well as in scrapie-sick RIPLTα mice. No prion infectivity was identified in any of the control animals at any time point after prion inoculation (**Figure 5**).

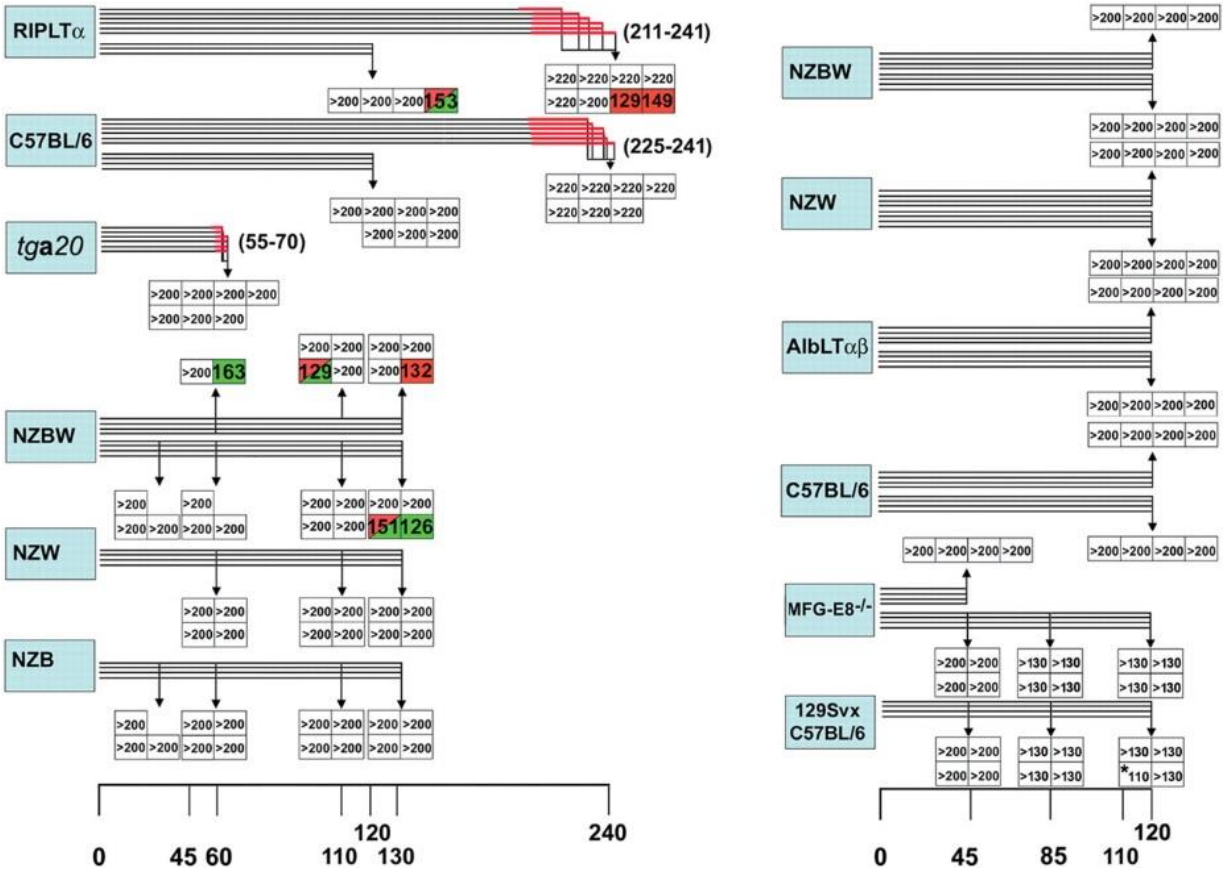

Figure 4. Transmission of prions through urine. Urine samples were collected from individual donors (horizontal lines) at time points after intraperitoneal prion inoculation, denoted by vertical lines, and pooled (intersections between lines, arrows). Squares represent individual tga20 mice inoculated i.c. with urinary proteins. White squares: no scrapie symptoms; red squares: histopathologically confirmed scrapie; green squares: positive PrPSc immunoblot. Numbers within squares: days to terminal disease. Clinical disease: red line. Prion incubation time is expressed in days. Asterisk: intercurrent death without clinical scrapie signs.

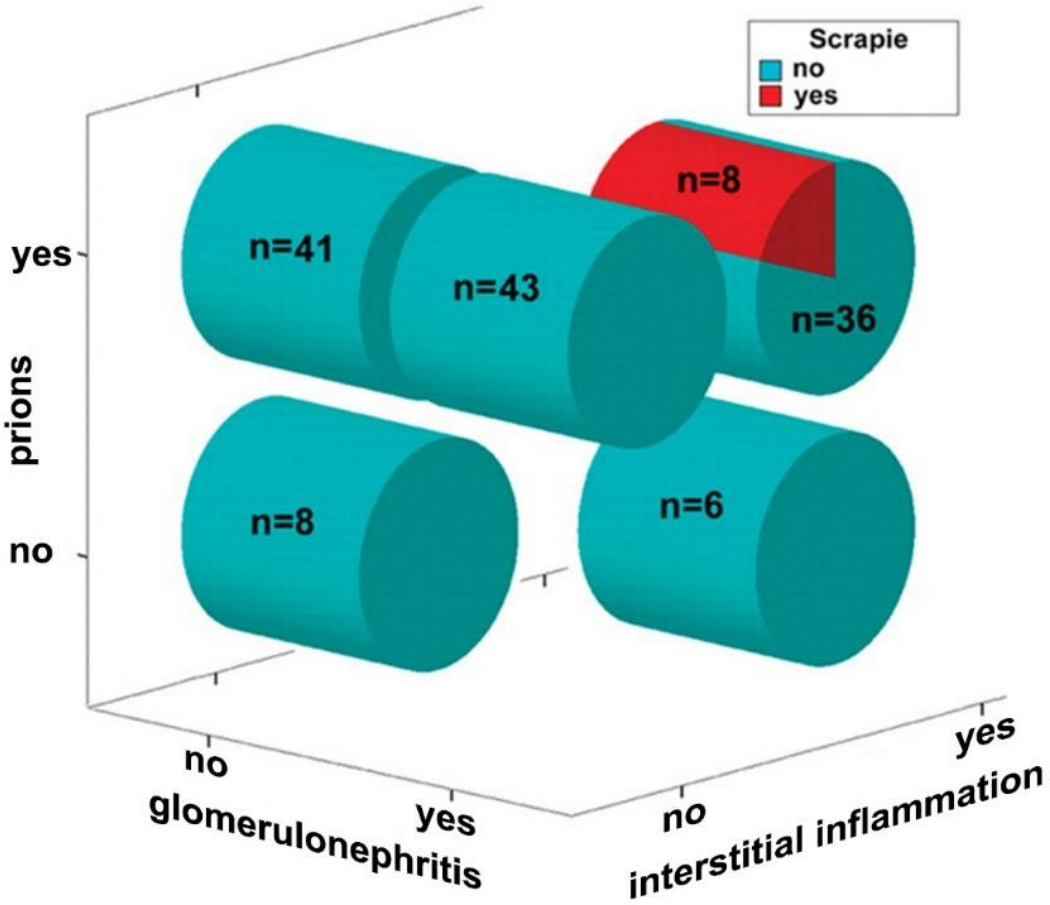

Figure 5. Prion infectivity in mouse urine as assessed by mouse bioassay. Prion infectivity was only detected in urine from animals, which were infected with prions and had follicular interstitial inflammation (18.2%) but not in urine from mice without kidney pathology or with isolated glomerulonephritis.

Whereas RIPLTα and NZBW mice suffer from combined interstitial lymphofollicular inflammation and glomerulonephritis, MFGE8 -/-, NZW, and NZB mice display glomerulonephritis but lack interstitial inflammation. We conclude that prionuria necessitates intrarenal organized inflammatory foci but not isolated glomerulonephritis. How do prions enter the urine? Blood-borne prions may be excreted by a defective filtration apparatus upon extrarenal replication. Alternatively, prions may be formed locally and excreted during leukocyturia. Although prionemia occurs in many paradigms of peripheral prion pathogenesis[81, 82], the latter hypothesis appears more likely, because prionuria in our study was invariably associated with local prion replication within kidneys.

2.3 The lymphotoxin β receptor is a therapeutic target in renal inflammation[83]

Little data are available on the LT pathway in human kidney diseases. Tubulointerstitial lymphoid infiltrates are often present in chronic renal diseases. As the LT pathway is known to be involved in chronic inflammation and the formation of TLOs[70], we hypothesized that LTβR signaling plays a role in human renal diseases. Thus, we evaluated the expression of LTs and LTβR in human renal biopsies, the effects of LTβR signaling in renal cells in vitro, and the effect of LTβR inhibition in a rodent lupus model. We considered the involvement of the LT pathway in settings both with and without TLO involvement.

In GN, injury begins in the glomerular tuft and with progression, inflammation and fibrosis spreads to the tubulointerstitium. To define a potential role for LTβR signaling, we performed transcriptional profiling of glomerular and tubulointerstitial compartments from renal biopsies with lupus nephritis (LN) and immunoglobulin A nephropathy (IgAN) using cDNA microarrays. In both diseases, LTβ mRNA was significantly upregulated in the tubulointerstitium, as well as in glomeruli. LTβR and LTα were expressed and LTα mRNA levels correlated significantly with the expression of LTβ. LIGHT mRNA was also increased in glomeruli. Expression of several genes known to be regulated by LTβR signaling was increased, notably, the chemokines CXCL12, CCL19, and CCL21.

mRNA expression of LTβ in renal biopsies was confirmed in a second cohort by qRT-PCR. LTβ mRNA expression was significantly higher in the tubulointerstitium from patients with LN as compared with controls. The same was true in crescentic and IgA glomerulonephritis (**Figure 6)**. In individuals with GN, LTβ protein was present on infiltrating inflammatory and renal tubular epithelial cells (TECs) of the proximal tubule. Parietal epithelial cells (PECs) and cells in crescents were sometimes positive for LTβ in proximity to the corresponding receptor. Nodular accumulations of infiltrating cells were present in 24% and these contained higher scores for LTβ-positive lymphocytes. The LTβ receptor was widely expressed by intrinsic renal cells. In glomeruli, LTβR protein and mRNA were present on parietal epithelial cells (PECs) and in the mesangium. Prominent expression was detected on cells in extracapillary proliferations in crescentic GN. TECs were commonly positive for the LTβR (**Figure 6**). In summary, the induction of LTβ in human renal biopsies occurred primarily in lymphocytes and to a lesser extent in TECs. The corresponding receptor was present mainly on intrinsic renal cells.

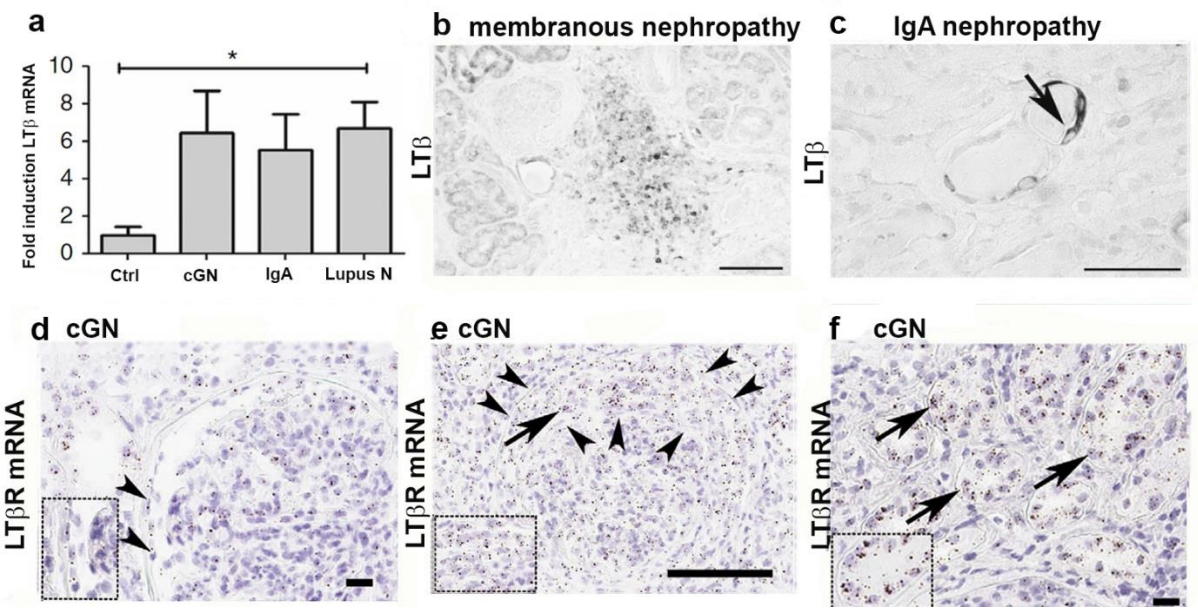

Figure 6. LTβ and LTβR in human renal biopsies with glomerulonephritis. (**a**) Expression of LTβ mRNAs were quantified by qRT-PCR in the tubulointerstitial compartment of renal biopsies from living donors (controls, Ctrl), and from patients with glomerulonephritis (*P < 0.05). (**b**, **c**) Immunohistochemistry on sections from renal biopsies using a monoclonal antibody against LTβ. (**b**) A structured interstitial infiltrate. The majority of cells in this lymphoid aggregate is LTβ-positive. (**c**) Tubular epithelial cells positive for LTβ (arrow) in a biopsy with IgA nephritis. (**d** - **f**) LTβR mRNA was localized in renal biopsies by in situ hybridization. (**d**) LTβR mRNA expression in PECs (arrowheads) and glomerular cells in a biopsy from cGN. (**e**) In addition to glomerular cells and PECs (arrowheads) LTβR mRNA expression was detected in cells within crescents (arrows); (**f**) LTβR mRNA was abundant in tubular epithelial cells. (scale bar 10 µm in d, f; 100 µm in e); inserts illustrate original magnification x 60). cGN, crescentic GN; LTβ, lymphotoxin b; LTβR, lymphotoxin β receptor; RT-PCR, reverse transcription polymerase chain reaction, PEC, parietal epithelial cells. Scale bar 100 µm in b-d, f, 50 µm in e (adapted from[83]).

As LTs and the LTβR were present in human kidney diseases, we used human renal tubular (HK-2) and mesangial (HMC) cell lines to further characterize the induction of LTs and the response to various stimuli. By stimulation with TNFα alone, or in combination with interferon (INF) γ we observed a strong upregulation of LTα and LTβ in both cell lines with different kinetics. The LTβR was strongly expressed in both cell lines. Upon stimulation with an agonistic LTβR antibody, the expression of several cytokines such as CCL2 (**Figure 7**), CCL5 and CXCL8 was induced in these cells. In primary mouse parietal epithelial cells (mPECs) a prominent increase in LTβ mRNA was detected after exposure to TNFα. Stimulation with an agonistic LTβR antibody resulted in a strong CCL2 induction (**Figure 7**). These data illustrate that human TECs and mesangial cells as well as mouse primary parietal epithelial cells can express LTs upon exposure to TNF. Furthermore, signaling via LTβR can directly activate

chemokine expression in vitro, consistent with the potential for this pathway to promote renal inflammation.

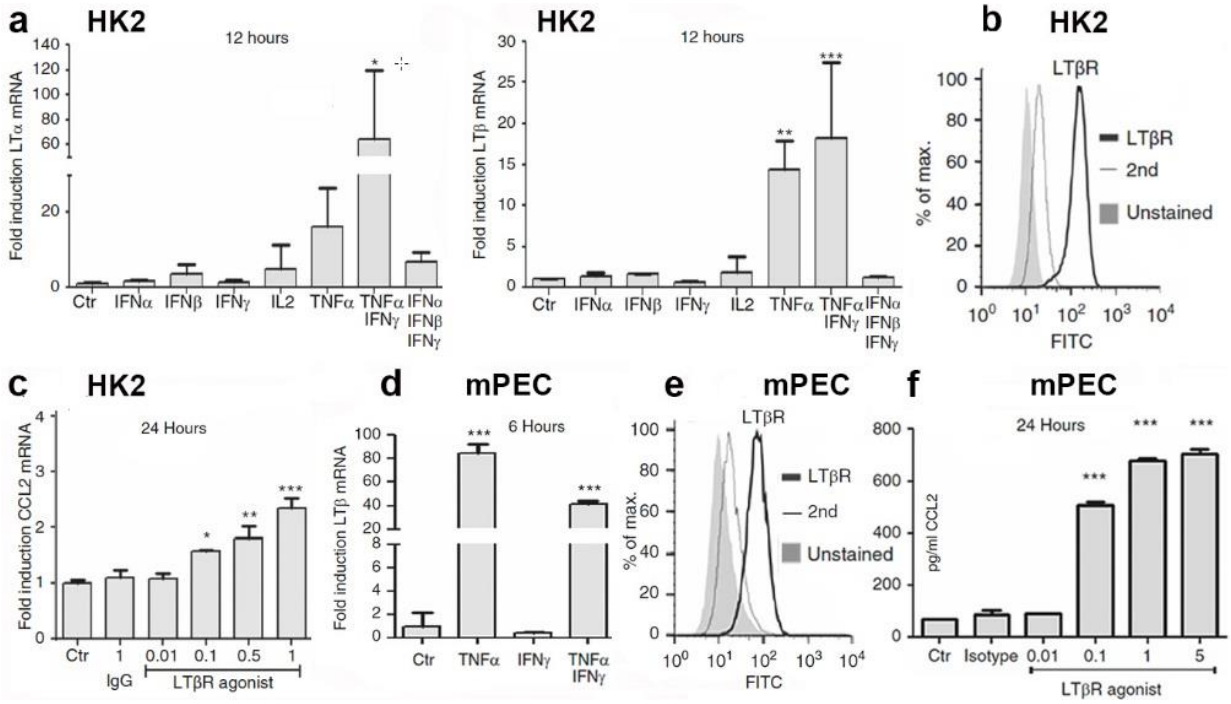

Figure 7. In vitro studies on human HK-2 cells (a-c) and mouse PECs (d-f) exposed to cytokines and a LTβR stimulating antibody. (**a**) HK-2 cells were exposed to cytokines IFNα, IFNβ, and IFNγ, a combination of all 3 IFNs, IFNαβγ (1000U/ml each), TNFα (25 ng/ml), a combination of TNFα and IFNγ, or to IL2 (5 ng/ml) for 12 hrs. LTα (left) and LTβ (right) mRNAs were quantified by real-time RT-PCR. (**b**) FACS analysis for the detection of LTβR on HK-2 cells (2nd: control with secondary antibody only, unstained: without antibodies). (**c**) HK-2 cells stimulated with an LTβR agonist. HK-2 cells were exposed to increasing concentrations of LTβR agonistic antibody (in mg/ml) or control IgG for 24 h. CCL2 was quantified by qRT-PCR (ctr: media control). **(d – f)** Activation of mouse primary PECs by cytokines and LTβR signaling. (**d**) PECs were exposed to medium (ctr), TNFα (25 ng/ml), IFNγ (1000U/ml) or a combination of TNFα and IFNγ. LTβ mRNA was quantified by qRT-PCR. (**e)** Expression of LTβR protein in PECs was confirmed by FACS analysis. (**f**) mPECs were exposed to increasing concentrations of LTβR agonist antibody, control IgG or medium. CCL2 protein quantified by ELISA demonstrates significant induction. IFN, interferon; IL, interleukin; LT, lymphotoxin; TNF, tumor necrosis factor; RT-PCR, reverse transcriptase polymerase chain reaction. FACS, fluorescence-activated cell sorting; LTβR, lymphotoxin β receptor. ELISA, enzyme-linked immunosorbent assay; PECs, parietal epithelial cells. (*P < 0.05, **P < 0.01, ***P < 0.001); (adapted from[83]).

Finally, we tested the efficacy of blocking the LTβR pathway in an accelerated mouse model of lupus nephritis. LTβR-Ig binds to LTα1β2 and LIGHT, which inhibits their interaction with the LTβR. NZB × NZW F1 mice (BWF1) develop an immune-complex GN, which reflects many aspects of human lupus nephritis[84, 85]. Adenoviral delivery of IFNα (Ad-IFN) to BWF1 mice accelerates the development of renal disease, however

without formation of TLOs[86] as compared to the non- accelerated BWF1 mice. Treatment with LTβR-Ig was started at different times after the initiating viral transfer, (0, 3, and 5 weeks). We performed detailed analysis on mice at 7 weeks, with treatment starting at 3 weeks. Expression of renal LTβ mRNA rose progressively from week 5 (**Figure 8**). In situ hybridization demonstrated LTβ mRNA expression in glomeruli, focal interstitial infiltrates, and TECs at 7 weeks. The LTβR was widely expressed in PECs, cells of the glomerular tuft, and tubular epithelial cells. LTβR-Ig treatment strongly reduced proteinuria and improved blood urea nitrogen at week 7. Histology of the glomeruli revealed less glomerular cellularity and matrix deposition with LTβR-Ig treatment and less tubulointerstitial inflammation (**Figure 8**). Neither IgM nor pan IgG anti-dsDNA antibody titers were reduced by LTβR-Ig treatment nor did it alter glomerular immune complex deposition. The chemokine genes CCL2, CCL20, and CXCL10 were downregulated upon LTβR-Ig treatment. Interleukin-6, which has been shown to correlate with lupus disease activity[87], correlated with proteinuria and was significantly reduced by LTβR-Ig treatment. Taken together, LTβ and LTβR are expressed in BWF1 mice consistent with the pattern presented for human glomerular diseases. LTβR-Ig treatment for 4 weeks protected mice from renal damage and improved renal function at a stage *after* disease initiation. Treatment was associated with the downregulation of major cytokines and chemokines, such as CCL2 but independent of immune complex deposition. Our data suggest that blocking LTβR signaling in this lupus model decreases chemokine and cytokine release from renal cells resulting in reduced proliferation of glomerular as well as a diminished influx of inflammatory cells from the blood thereby reducing renal damage.

In summary, we found that LT ligands are expressed in renal cells in vitro, in vivo, and in human renal biopsies. Renal cells can respond to LTβR activation with the potential to promote and amplify inflammation. Our data provides the first in vivo evidence for a role of LTβR signaling in lupus nephritis.

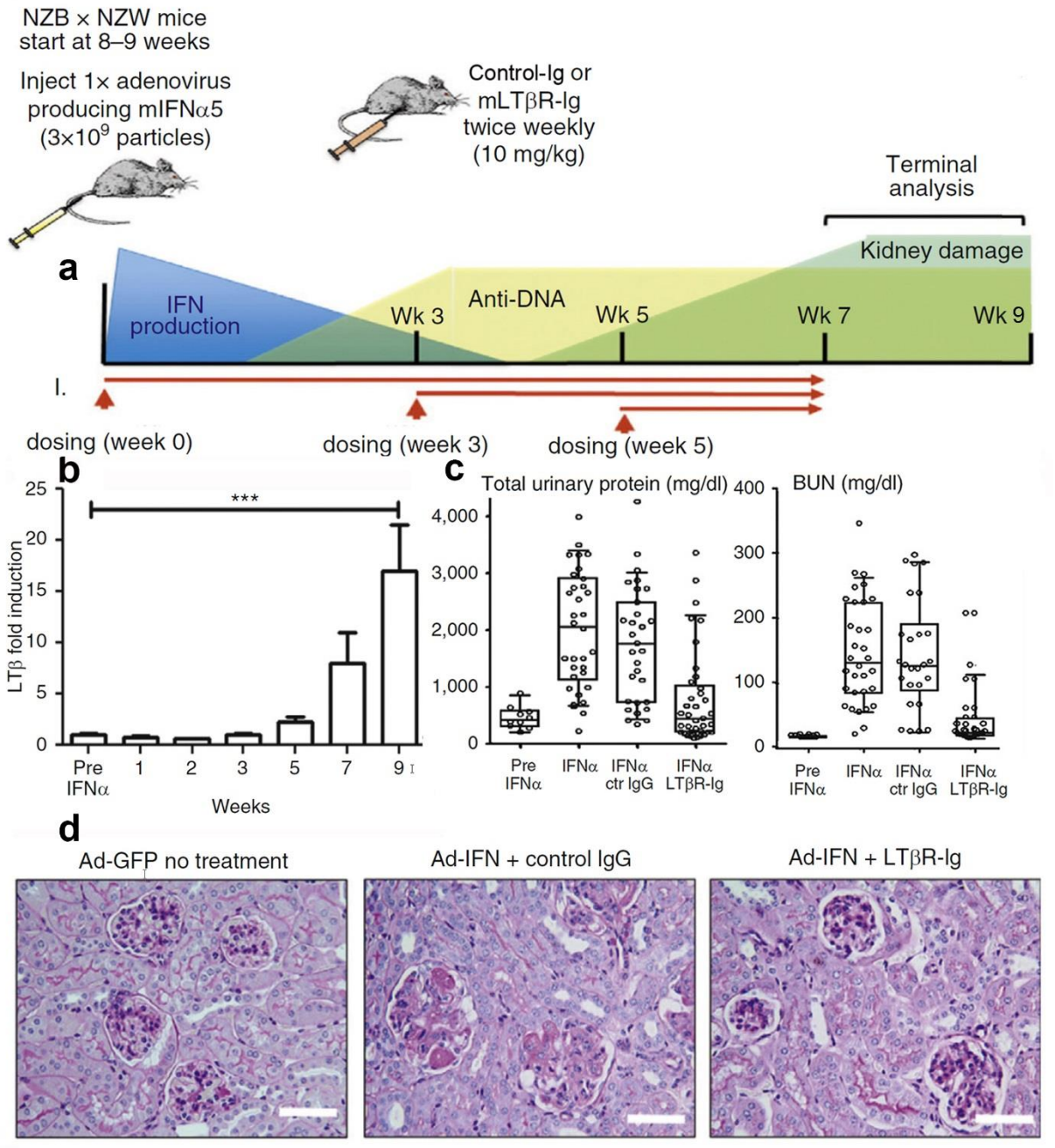

Figure 8. Treatment with LTβR-Ig reduces renal disease in the adenoviral-IFN accelerated BWF1 model. (**a**) An outline showing dosing regimens with arrows indicating the onset of drug treatments (mice treated between week 3 and 7 are illustrated in b-d). (**b**) Expression of renal LTβ mRNA measured by qRT-PCR illustrates a progressive increase after 5 weeks. (**c**) A significant reduction in urinary protein and BUN was detectable. Total urinary protein and BUN prior to infection with the adenovirus (Pre-IFNa) or 7 weeks after infection with no treatment (IFNα only), with a control antibody treatment (IFNα+control Ig) or with prophylactic LTβR-Ig treatment (IFNα+LTβR-Ig). Box and whisker presentation of the data (arms 10%–90% range) is shown with each circle representing one animal. Mann-Whitney comparison of control-Ig– and LTβR-Ig–treated groups was significant ($P < 0.0001$). (**d**) Treatment with LTβR-Ig resulted in a significant improvement of glomerular injury (periodic acid-Schiff stains, scale bars = 100 mm). Ad-GFP, Adenovirus green fluorescent protein (control adenovirus); Ad-IFN, adenovirus-interferon; BUN, blood urea nitrogen; IFN, interferon;

LTβ, lymphotoxin b; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction. (***P < 0.001); (adapted from [83]).

2.4 Lymphotoxin expression in human and murine renal allografts[88]

Acute and chronic renal rejection are inflammatory processes and in renal allograft biopsies TLOs are present in up to 50%[32-34]. Even though early studies have provided evidence for the expression of LTβ in rejected rat and human renal allografts[89, 90], a comprehensive investigation of LT expression in renal transplants has not been undertaken. Therefore, we examined the differential regulation of components of the LT system in human renal allograft biopsies and a murine model of renal transplantation.

cDNA microarray analysis of human kidney allografts demonstrated robust upregulation of LTβ, LIGHT and one of its receptors, HVEM, as well as TNF receptors 1 and 2 mRNA in kidneys with acute rejection (AR) compared to controls. Interestingly, in chronic allograft injury/IFTA (interstitial fibrosis/tubular atrophy) we observed a similar pattern. The upregulation of transcripts controlled by the alternative NF-κB signaling pathway, such as CXCL12, CXCL13, CCL19 and BAFF (B cell activating factor) in AR and IFTA strongly suggested activation of the LTα1β2-LTβR or LIGHT-LTβR axis since aside from CD40L-CD40 interaction and BAFF[91-94], LTβR activation is one of three major inducers of the alternate NF-κB pathway[95]. CD40L was neither upregulated in AR nor chronic allograft nephropathy and therefore unlikely to be solely responsible for the alternate NF-κB pathway triggering in this context. LTα and TNFα mRNAs were not significantly increased in the transcriptome of grafts with AR and IFTA compared to nephrectomy controls, which was unexpected, since activated lymphocytes as well as resident dendritic cells, have been shown to express these cytokines. LTβR mRNA did not show any differential regulation. This is in line with the previously described constitutive expression of the receptor in parenchymal cells and its promotor region suggesting a house keeping function[96].

In an independent set of 49 allograft biopsies analyzed by qRT-PCR, we detected robust upregulation of LTβ, and LIGHT in borderline rejection and AR. In addition, LTα mRNA was upregulated. In allografts with IFTA, we also noticed a trend towards higher expression of LTα, β and LIGHT, which, however, did not reach statistical significance. Concordant with the above findings, LTβR was not differentially regulated (**Figure 9**). Interestingly, also the decoy receptor 3 (DcR3) mRNA was significantly upregulated in AR. DcR3 is a secreted decoy receptor which binds to and blocks the biologic action of the ligands FasL and LIGHT[14, 15, 97, 98] but can also activate T cells via binding to TL1A[16]. It was recently shown to have negative prognostic value in patients with chronic kidney disease[99]. Whether DcR3 is a predictor of negative outcome also in renal transplant patients needs to be determined. There was no significant expression difference between borderline rejection and AR with respect to the transcripts

investigated. This could indicate that despite a morphologic difference on the light microscopic level acute and borderline rejection share features on the transcriptional level. Another possible explanation is that some of the cases diagnosed with borderline rejection might in fact have been cases of acute rejection. In our study, most of the cytokine mRNA levels in the different subgroups (borderline, AR and IFTA) did not significantly correlate with the degree of histopathological changes in the different renal compartments. This is in line with previous studies, which demonstrated that transcriptional upregulation of inflammatory or injury associated genes do not correlate well with morphological changes in transplant biopsies but often predict the outcome better than the histology[100, 101].

In a further experimental paradigm, mRNA expression patterns of members of the LT family and structurally related genes were assessed using cDNA arrays in patients with another chronic renal allograft injury termed transplant glomerulopathy (TG). Clustering analysis showed two distinct expression patterns. One cluster displayed strong upregulation of LTα, TNF, LIGHT, HVEM, BTLA, CXCL13, CCR7 and CCL21 (cluster 1), whereas the second pattern (cluster 2) was characterized by increased expression of the LTβR, LTβ, TNF receptors 1 and 2, MADCAM and TROY. The segregation of these two clusters could reflect different progression stages of TG or distinctive underlying pathologies characterized by engagement of a different subset of components of the LT and TNF system resulting in a similar histopathologic pattern. The clusters might also reflect different stages of chronic interstitial inflammation with formation of ectopic lymphoid follicles.

To investigate the expression of LTβ on the protein level, we performed immunohistochemistry on allograft biopsies. The analysis exhibited LTβ immunoreactivity in inflammatory cells and tubular epithelial cells (TECs) in biopsies with AR and IFTA (**Figure 9**). In implant biopsies, we rarely observed LTβ immunoreactivity. As in kidneys with various forms of GN (see 2.3), we observed expression of LTβ on infiltrating mononuclear inflammatory cells diffusely within the tissue or in follicular infiltrates. The observed upregulation of LTβ in TECs suggests that tubular damage or TEC activation by cytokines from infiltrating cells leads to upregulation and release of LTβ, which may activate the LTβR on neighboring TECs and/or local immune cells such as DCs, modulating the inflammatory response. That TECs serve as regulatory cells in acute rejection – either amplifying or ameliorating inflammation – has been shown in previous studies[102]. Interestingly, we did not observe tubular expression of LTβ in implant biopsies. This indicates that even a severe ischemic stimulus (cold and warm ischemia) does not suffice to induce LTβ expression in TECs.

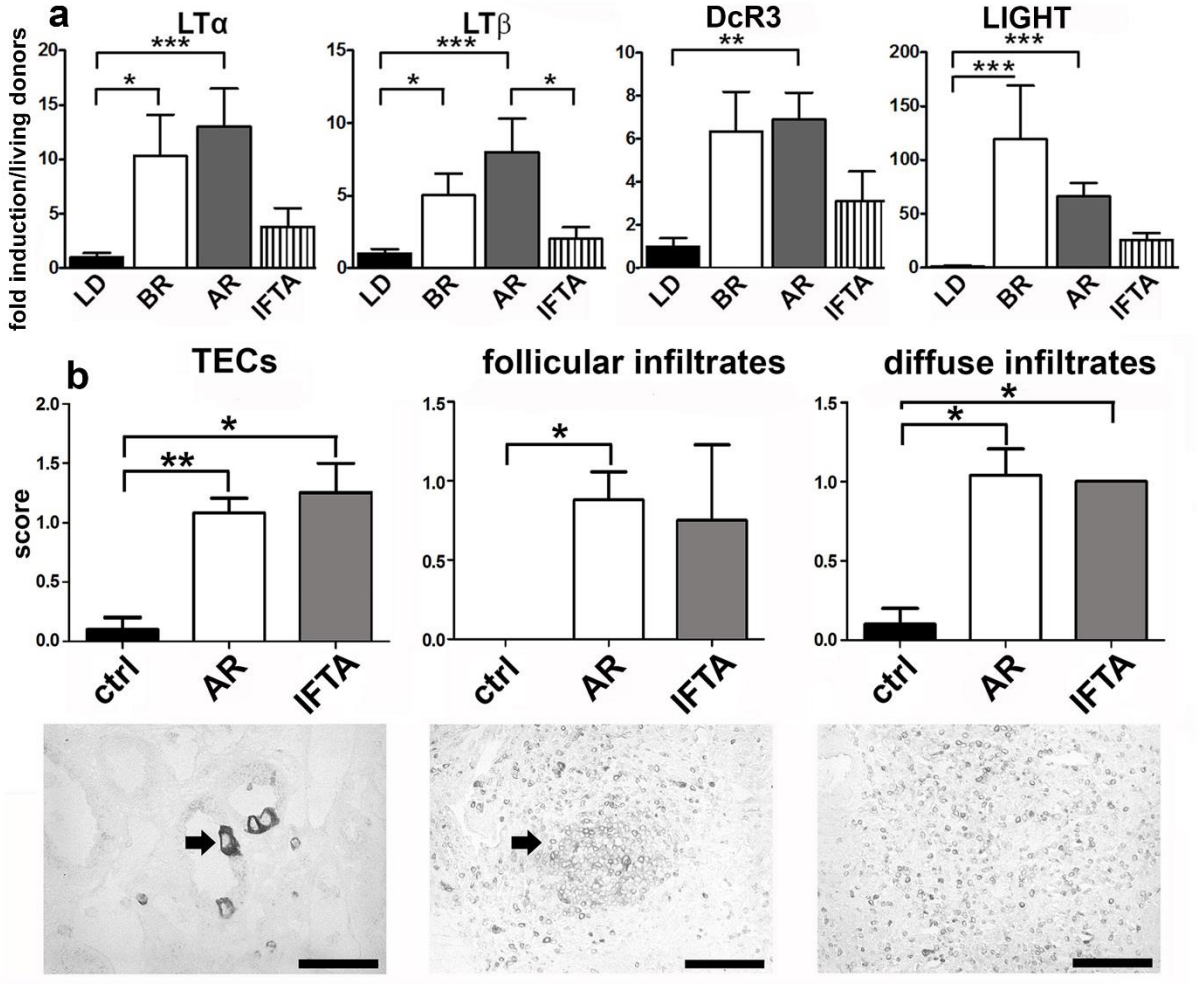

Figure 9. Expression of LTs in human renal allografts. (**a**) Upregulation of LT mRNAs in human renal allograft biopsies compared to biopsies from living donors. LTα, LTβ, LTβ receptor, DcR3 and LIGHT mRNA expression was quantified by qRT-PCR. Control biopsies were taken from living donors (LD) before implantation (displayed are mean scores + SEM). (**b**) Semiquantitative expression of LTβ in human allograft biopsies. LTβ immunohistochemistry was performed in biopsies with AR, IFTA and controls (ctrl). Upper panel shows semiquantitative analysis of LTβ positivity in tubular epithelial cells (TECs; left), LTβ positivity in infiltrating inflammatory cells in follicular infiltrates (middle) and LTβ positivity in diffusely infiltrating inflammatory cells (right). The lower panel shows examples of LTβ positivity in TECs (left; arrow depicts positively stained tubular epithelial cell), follicular infiltrates (middle; arrow depicts follicular infiltrate) and diffusely infiltrating cells (right) (scale bar = 50 µM). BR borderline rejection, AR acute rejection, IFTA interstitial fibrosis/tubular atrophy, LTβ, lymphotoxin β; LTβR lymphotoxin β receptor; TNFR, tumor necrosis factor receptor (* $p< 0.05$; ** $p < 0.01$, *** $p<0.001$); (adapted from[88]).

To explore the expression of LTs in renal transplants in another species we employed a model of acute renal allograft rejection using two fully MHC mismatched mouse strains[103]. In this model, we recapitulated our findings from human biopsy studies. Seven days after orthotopic renal grafting without immunosuppressive treatment histology of allografts showed severe acute tubulointerstitial rejection with interstitial

edema, diffuse inflammatory infiltrates, tubulitis and signs of acute tubular injury. qRT-PCR displayed significant induction of LTα, LTβ, HVEM and LIGHT mRNAs. Again LTβR was not differentially expressed (**Figure 10**). These findings indicate that with respect to the LT system, mice react in a similar fashion as humans to engraftment with a foreign kidney. Given the ample disparities between the human and mouse immune system, this finding is critical regarding the translatability of results from studies in renal transplantation using this model system.

To summarize, our study demonstrates for the first time, that essentially all components of the LT system are expressed and a subset of them strongly upregulated in rejected human renal allografts and in the acutely rejected mouse kidney. We therefore provide evidence for the involvement of the LT system in acute kidney rejection and chronic allograft injury.

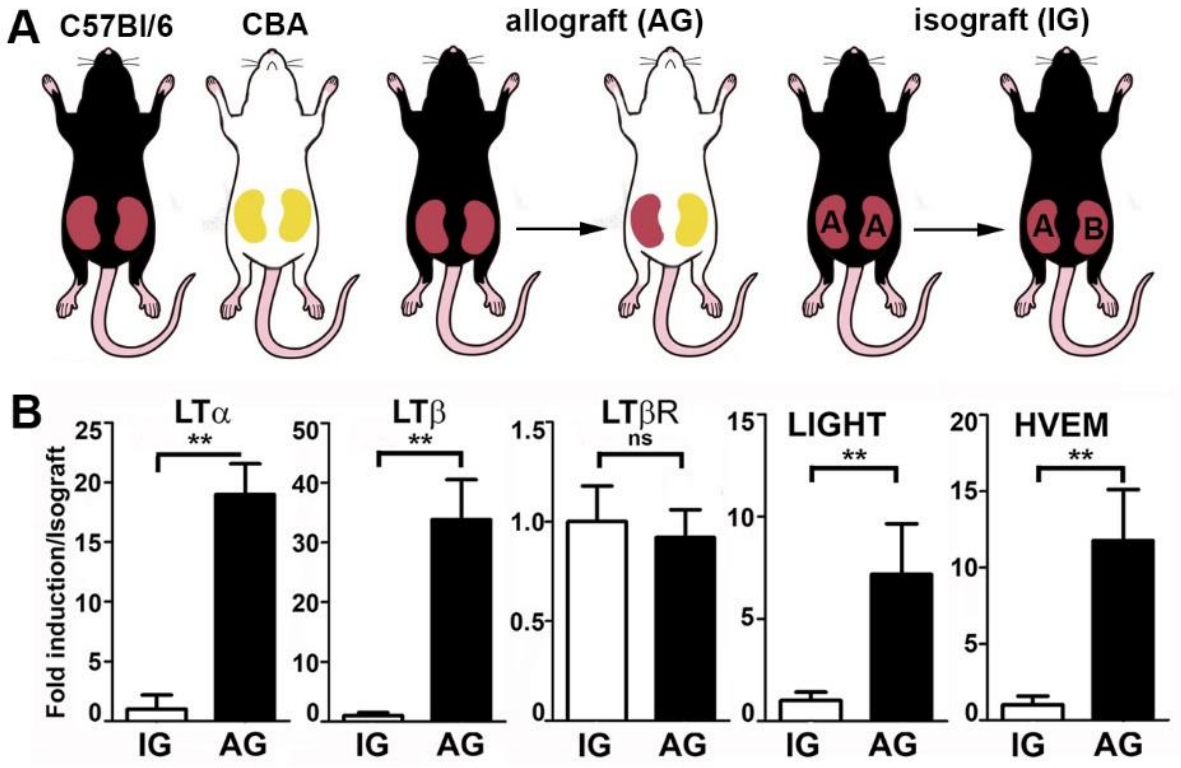

Figure 10. Lymphotoxin mRNA expression in mouse renal allografts. (**A**) Kidneys were transplanted from C57BL/6 into CBA mice (fully MHC mismatched allografts) or into C57BL/6 (isografts). Kidneys were removed after seven days without any immunosuppressive treatment. (**B**) Mean fold-induction of LTα, LTβ, LTβ receptor, HVEM and LIGHT mRNAs quantified by quantitative RT-PCR for allografts compared to isografts seven days post transplantation. A very significant induction was found for LTα, LTβ, HVEM, LIGHT. (* $p < 0.05$, ** $p < 0.01$, *** $p<0.001$, ns = non significant.)

3. Conclusion and Outlook

Taken together, the first part of the work described above (2.1 and 2.2) demonstrated that chronic inflammation with TLO like structures permits accumulation of prion infectivity in normally prion-free organs. Tissue-specific expression of the proinflammatory cytokine (LTα) or chemokine (SLC) resulted in the establishment of new prion reservoirs. LTs therefore seem to play a major role in prion replication not only in SLOs, but also in TLOs. Furthermore, we found that inflamed kidneys of prion infected animals elicited prionuria. Our study was the first in the literature to prove that prion infectivity can occur in urine of animals with prion disease.

What is the relevance of these findings? Knowing the tissue distribution of prion infectivity within infected individuals is crucial for the prevention of iatrogenic spread of prion diseases. In addition, there are important implications for the food industry and consumer protection with respect to transmission of bovine spongiform encephalopathy. Up to the point of our study, the risk of transmitting TSEs from tissues other than central/peripheral nervous system, lymphatic or intestinal tissues has been considered small[104]. However, our data suggest that extraneural and non-lymphoid tissues might gain the ability to accumulate or replicate prion infectivity if they are affected by chronic inflammatory conditions. Our findings furthermore indicate, that urine of individuals with prion disease such as CJD has to be considered potentially contagious. Prions excreted via urine could lead to prion contamination of the soil. Inflammation-associated prionuria may contribute to TSE transmission among sheep and free ranging deer and elk.

Interestingly, a subsequent study demonstrated, that in sheep with concomitant scrapie infection and chronic lymphocytic mastitis, accumulation of PrP^{Sc} occurred in inflammatory follicles in mammary glands[105]. Mothers with mastitis and scrapie passed the disease on via milk to most of the suckling lambs[106]. Another successive study reported prion infectivity in urine from free ranging deer with chronic wasting disease (a prion disease affecting cervids). Lymphocytic inflammation was detected in the kidneys in 4/5 animals with infectious urine[107].

Our further studies on inflammatory kidney diseases and renal allograft rejection (2.3 and 2.4) suggest that LTs may be important players in these conditions. We demonstrated, that LTβ is not only expressed by infiltrating cells, but also by intrinsic renal cells such as tubular epithelial cells. Our experiments in cultured immortalized and primary renal cells indicate that LTβR signaling appears to be a new pathway for the activation of renal cells and amplification of inflammation within the kidney. Since the lupus model we used did not involve TLOs, yet LTβR blockade still alleviated disease, interfering with LT signaling might be helpful in renal inflammatory conditions with and without TLO formation. Because in humans with lupus nephritis the disease is resistant to current immunosuppressive therapy in 20% to 70% of affected

individuals[108], novel therapies are urgently needed. We think that blocking LTβR signaling could be a promising therapeutic approach in human lupus nephritis, but also other forms of chronic renal inflammation such as IgA nephropathy or crescentic glomerulonephritis.

From our data, we cannot deduce which components of the LT system serve as positive or negative regulators in renal inflammation. Whereas inhibition of LTβR signaling seems to be beneficial in lupus nephritis, the role of LTs in allograft rejection is unclear. On the one hand, interfering with the LT system could be beneficial. In murine antibody mediated cardiac allograft rejection, blocking of the LTβR resulted in complete abrogation of TLO formation and prevented rejection[109]. Conversely, LTβR blockade in another model broke tolerance and caused inflammation and fibrosis of cardiac allografts[110]. We reason that LTβR blockade has the potential to be beneficial especially in chronic antibody mediated rejection (cABMR), for which efficient treatments are lacking[111]. An interesting study by Thaunat and colleagues examined explanted allografts of patients suffering from cABMR who had received B cell depleting therapy (rituximab). Despite complete absence of circulating B cells TLOs were detected within the graft with B cells persistently producing alloantibodies[112]. Given the capacity of LTβR blockade to abrogate TLOs[109] we plan to examine the therapeutic potential of blocking LT signaling in a mouse model of cABMR with intra-graft TLO formation[113]. However, as demonstrated above in the IFN–accelerated BWF1 lupus mouse, LTβR blockade can also alleviate renal inflammation without TLO formation. Therefore, we intend to investigate LTβR blockade using sLTβR-Ig and a LTα neutralizing antibody in our model of acute murine allograft rejection. Several available (conditional) knockout models for various components of the LT system will aid us in dissecting the exact contribution of the LT system to renal allograft rejection.

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